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CYP2C19 POLYMORPHISMS IN DRUG METABOLISM AND RESPONSE

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CYP2C19 polymorphisms in drug metabolism and response
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There is in fact a true law - namely, right reason - which is in accordance with nature, applies to all men, and is unchangeable and eternal.

Marcus Tullius Cicero

ABSTRACT

Individuals vary widely in their response to drug treatment. After receiving doses of a drug that are recommended based on a population average, some patients could have an insufficient response, whereas others may experience adverse effects. Of the many factors causing variability in drug response across individuals, genetic polymorphism of drug-metabolizing enzymes is deemed to be one of the valuable independent predictors of this variability. CYP2C19 is an important polymorphically expressed enzyme known to catalyze the metabolism of several widely prescribed drugs, including omeprazole, warfarin (R-enantiomer), and citalopram/escitalopram. The impact of CYP2C19 polymorphisms on the pharmacokinetics (PK) of its substrates and corresponding clinical relevance are of great interest.

The aim of this thesis is to investigate the influence of CYP2C19 polymorphisms on PK and pharmacodynamics (PD) of clinically important CYP2C19 substrates (e.g., omeprazole, citalopram, and warfarin), and to advance the understanding of their inter-individual variability in drug therapy. We quantified the effect of functional *CYP2C19* allele variants, including the gain-of-function allele (*CYP2C19*17*), on drug exposure and response in order to facilitate personalized dose selection.

In Paper I, we studied the disposition of omeprazole and its effect on plasma gastrin levels following single and multiple doses in the three phenotype groups (extensive metabolisers [EMs], intermediate metabolisers [IMs], and poor metabolisers [PMs]) of S-mephenytoin hydroxylation. When 20 mg of omeprazole was given orally for 8 days on a once-daily (QD) regimen, the relative AUC ratios in EMs, IMs, and PMs were 1:5.3:13.1. Differences in the plasma gastrin levels (used as a PD marker) were also significant between the three groups and the increase was in an omeprazole-concentration-dependent fashion. Suitability of omeprazole as a probe for CYP2C19 was also explored. The metabolic ratio (MR) of omeprazole was correlated significantly with S/R ratio of mephenytoin.

Paper II further studied the use of omeprazole as a probe for CYP2C19 activity in a population of 160 unrelated Swedish subjects. There was a close correlation between MRs of omeprazole and S/R ratios of mephenytoin. A good agreement was also demonstrated between the CYP2C19 phenotypes (both by omeprazole and mephenytoin) and the genotype with respect to *CYP2C19*2*, indicating that genotype is a valid predictor of CYP2C19 activity. Since omeprazole is a substrate for both CYP2C19 and CYP3A4, the potential advantage also includes using it as a dual-substrate probe.

In Paper III, we used omeprazole as a dual substrate probe to assess the potential for PK interactions between carbamazepine (CBZ) and omeprazole and, particularly, the inducibility of CYP3A4 and CYP2C19 by CBZ. Both omeprazole and hydroxyomeprazole decreased by approximately 40% in mean AUC after coadministration of omeprazole with CBZ, while the sulphone metabolite increased by 44%. None of the AUC changes were statistically significant due to the large variation and small sample size. A significant decrease in the AUC ratio between hydroxyomeprazole and sulphone metabolite was observed, suggesting induction was more pronounced for CYP3A4 than for CYP2C19.

The potential contribution of the CYP2C19 genotypes on R-warfarin clearance with special focus on the gain-of-function allele (*CYP2C19*17*) was the primary objective for Paper IV. Compared to *CYP2C19*2* carriers, the mean R-warfarin clearance increased by 32% in *CYP2C19*17* carriers, 26% in the *CYP2C19*2/*17* genotype, and 11% in *CYP2C19*1/*1* genotype. *CYP2C19* genotypes also contributed to the variability of INR/daily dose where *VKORC1* (Vitamin K epoxide reductase subcomplex 1) and *CYP2C9* genotypes are the major determinants in warfarin treatment. About 52% of variance can be explained by the combinations of *VKORC1*, *CYP2C9*, *CYP2C19*, age, gender, and bodyweight, of which *CYP2C19* genotypes accounted for 7%.

Paper V pooled data from16 published studies to quantify the effect of functional *CYP2C19* allele variants on citalopram/escitalopram exposure by means of meta-analysis. Compared to subjects with EM/EM (**1/*1*) genotype, the exposure to (es)citalopram increased by 95% in the PM/PM (**2/*2, *2/*3,* or **3/*3*), 30% in the EM/PM (**1/*2* or **1/*3*), and 25% in the UM (ultrarapid metaboliser)/PM (**17/*2* or **17/*3*) groups. In contrast, the exposure to (es)citalopram decreased by 36% in the UM/UM (**17/*17*) and by 14% in the UM/EM (**17/*1*) groups. All functional *CYP2C19* genotypes showed significant effects on citalopram/escitalopram exposure compared to the *CYP2C19*1/*1* genotype.

In conclusion, there are significant effects of CYP2C19 polymorphisms on PK and PD of drugs that are metabolized by the CYP2C19 enzyme. The results of this thesis demonstrate that *CYP2C19* genotype is an important independent predictor of the exposure to omeprazole, R-warfarin, and citalopram/escitalopram. Increased knowledge and understanding of inter-individual variability, genotype-phenotype correlation, and the impact of CYP2C19 polymorphisms on clinical practice are helpful in optimizing personalized drug therapy.

LIST OF SCIENTIFIC PAPERS

I. **Chang M**, Tybring G, Dahl ML, Götharson E, Sagar M, Seensalu R, Bertilsson L. Interphenotype differences in disposition and effect on gastrin levels of omeprazole - suitability of omeprazole as a probe for CYP2C19. Br J Clin Pharmacol. 1995; 39(5): 511-8.

II. **Chang M**, Dahl ML, Tybring G, Götharson E, Bertilsson L. Use of omeprazole as a probe drug for CYP2C19 phenotype in Swedish Caucasians: comparison with s-mephenytoin hydroxylation phenotype and CYP2C19 genotype. Pharmacogenetics 1995; 5: 358-63.

III. Bertilsson L Tybring G, Widen J, **Chang M**, Tomson T. Carbamazepine treatment induces the CYP3A4 catalyzed sulphoxidation of omeprazole, but has no or less effect on hydroxylation via CYP2C19. Br J Clin Pharmacol. 1997; 44(2): 186-9.

IV. **Chang M**, Söderberg MM, Scordo MG, Tybring G, Dahl ML. *CYP2C19* genotypes including *CYP2C19*17* affect R-warfarin plasma clearance and dose-INR relationship in patients treated with warfarin. [Submitted to Eur J Clin Pharmacol]

V. **Chang M,** Tybring G, Dahl ML, Lindh JD. Impact of CYP2C19 polymorphisms on citalopram/escitalopram exposure: a systematic review and meta-analysis. Clin Pharmacokinet 2014; 53(9): 801-11

CONTENTS

LIST OF ABBREVIATIONS

ADME	Absorption, distribution, metabolism, and excretion
CAR	Constitutive androstane receptor
CBZ	Carbamazepine
CL	Clearance
CNV	Copy-number-variation
CV	Coefficient of variation
CYP	Cytochrome P450
DDI	Drug-drug interaction
DNA	Deoxyribonucleic acid
EM	Extensive metaboliser
ESE	estrogen response element
FDA	Food and drug administration
HPLC	High-performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
IM	Intermediate metaboliser
MR	Metabolic ratio
NR	Nuclear receptors
PCR	Polymerase chain reaction
PD	Pharmacodynamics
PK	Pharmacokinetics
PM	Poor metaboliser
PPI	Proton pump inhibitor
PXR	Pregnane X receptor
miRNA	Micro messenger ribonucleic acid
SNP	Single nucleotide polymorphism
$T_{1/2}$	Half-life
UM	Ultrarapid metaboliser
VKOR	Vitamin K epoxide reductase
VKORC1	Vitamin K epoxide reductase subcomplex 1

1 INTRODUCTION

PRIMER

In early spring of 1992, Professor Ya-qing Lou at Beijing Medical University and her group collaborated with a research team at Karolinska Institutet led by Dr. Leif Bertilsson. The goal of his project was to compare debrisoquin and S-mephenytoin hydroxylation between two large population samples of native Chinese and Swedish healthy subjects. Being an associate researcher at the time, I assisted Prof. Lou in recruiting healthy volunteers. It turned out that two of my close family members participated in the study and both were identified as PMs of S-mephenytoin. This early touch on the concept of CYP2C19 polymorphism at least partially accounts for my long journey into this interesting area of research.

1.1 DRUG METABOLISM AND CHARACTERIZATION

Most drugs are primarily eliminated through metabolism processes. Drug metabolism is an enzymatic conversion of one chemical compound into another [1]; the term "metabolite" describes the product formed by metabolism. The metabolism process is commonly divided into two phases of biochemical reactions, i.e. Phase 1 and Phase 2 reactions. Phase 1 reactions transform the parent drug into a more polar metabolite by oxidation, reduction, or hydrolysis, a vital step before the drug can be excreted into the urine or bile. Inactive prodrugs can also be converted to the active molecule through Phase 1 reaction. Therefore, metabolites might be pharmacologically and toxicologically inactive, less active, equally active, or more active than the parent molecule. Phase 2 reactions, on the other hand, couple the drug or its polar metabolite with an endogenous substrate (for example UDP glucuronic acid, amino acid, sulfate, or acetate). Liver is the main organ for drug metabolism, but the metabolism processes can also occur in other organs, such as the intestines, kidney, and lungs.

To characterize the metabolic clearance of drugs, the pharmacokinetic (PK) properties of parent drugs and their metabolite(s), and the potential for metabolism based drug-drug interactions (DDI), a series of drug metabolism studies are usually conducted with a combination approach of *in vitro* and *in vivo* experiments [2].

1.1.1 Prediction of metabolic clearance using *in vitro* approach

In vitro experiments provide valuable information to the clearance mechanism for drugs and drug candidates. In the past decades, the knowledge of drug-metabolizing enzymes has increased tremendously so that prediction of the metabolic fate of drug candidates in human becomes possible. Typically, the *in vitro* experimental methods refer to the incubation with human liver microsomes, recombinant single expressed human isoforms (expressed enzymes), and cryopreserved or freshly isolated hepatocytes. The approach is employed to address a variety of questions, such as which enzymes are responsible for the metabolism of the drug? Is the drug candidate metabolically stable? Is there any human unique metabolite which was not tested in animal toxicity studies?

Metabolic stability, inhibition of drug-metabolizing enzymes and identification of specific enzymes responsible for the metabolism of a drug are the classic examples of utilizing *in vitro* approaches. To predict the clearance in humans and to understand the contribution of individual enzymes to the total clearance, the drug candidates can be incubated with single expressed human enzymes, pooled human liver microsomes, or hepatocytes with and without enzyme-specific chemical inhibitors. Metabolic clearance (CL_{int}), obtained based on the disappearance of the parent drug from the incubation mixture over incubation time, is used to estimate the hepatic clearance. The extrapolation from *in vitro* to *in vivo* is performed by factoring in amount of enzyme/tissue used in the incubation and the weight of the liver, assuming the well-stirred model for hepatic clearance. If the concentrations of test compound rapidly decline over time in systems expressing a specific enzyme (i.e. showing a high CL_{int}) and the reaction can be inhibited by an established chemical inhibitor, it is likely that the enzyme plays a role in the metabolism of the compound tested. Consequently, further evaluation of this compound in clinical setting would be considered.

1.1.2 Evaluation of drug metabolism using *in vivo* approach

Clinical evaluations are important to fully characterize the drug metabolism in humans. The metabolic profiles and elimination pathways are routinely generated from ADME (absorption, distribution, metabolism, excretion) studies in healthy subjects using radio-labeled drug. The ADME study also provides information on the total radioactivity recovered in urine, feces, and bile (known as mass balance) as well as quantitative

metabolites measurement relative to the parent drug in several matrices, typically in blood, urine, and bile.

Clinically relevant DDIs have been acknowledged as a cause of large inter-individual variability in PK and drug response. According to Furuta *et al* [3], AUCs of omeprazole increased 2.1-2.3 fold after coadministration of omeprazole with clarithromycin. The two drugs are frequently prescribed together to treat patients who have *Helicobacter pylori* infection and duodenal ulcer. When investigating DDIs in clinical trials, a typical study design is open-label, single-dose (multiple-dose for inducer test), two-way interaction in healthy subjects. The established substrates, inhibitors and/or inducers for the enzyme in question can be used as probe drugs in DDI evaluation.

1.1.3 *In vitro-in vivo* correlation

Human liver microsomes and hepatocytes have been reliably used to study inhibition mechanism and to predict human clearance. Due to various reasons, the discrepancy between *in vitro* and *in vivo* happens. For example, if standard assumptions that used for *in vitro* incubation are no longer applicable in humans, poor prediction outcome would be anticipated. Overall, whether or not to conduct an *in vivo* study is based on the quantitative measurement of the enzyme contributed to one pathway and the portion of metabolite formed from that particular pathway. The decision tree for metabolism-based-DDI-study, presented by US Food and Drug Administration (FDA) [4], is an example of a comprehensive approach incorporating cutting edge technology of *in vitro* experiments, modeling, and clinical trials.

The common practice for estimating DDI liability in human is to start with assessing the worst case-scenario by coadministration of the drug studied with a strong inhibitor. If the exposure to the drug with or without coadministration (e.g. AUC ratio) is within the predefined confidence interval of 0.8-1.25, the absence of a DDI for the metabolic pathway is demonstrated. On the contrary, a positive finding, i.e., confidence interval outside pre-specified interval, may lead to dosage adjustment, restrictions and cautions to concomitant use, additional therapeutic monitoring, or other measures to mitigate risk. As an alternative, physiologically-based pharmacokinetic (PBPK) modeling can be employed to simulate the trial and predict the clinical outcomes [4].

1.2 METABOLIC ENZYMES

The number of enzymes involved in drug metabolism reactions is large and govern catelyzing phase 1 oxidation, reduction, and hydrolysis reactions. Of the enzymes playing a role in the biotransformation of various pharmaceutical products, the dominant enzyme system is cytochrome P450 (CYP450) superfamily, accounting for 75% of reactions. UDP-glucuronosyl-transferases catelyze ~20% of the reactions.

1.2.1 Cytochrome P450 and nomenclature

Many cytochrome P450 enzymes have specific roles in the metabolism of steroids, eicosanoids and fat-soluble vitamins while about 1/4 of CYP450 enzymes are involved in the metabolism of xenobiotics including many drugs. CYP450 enzymes convert lipophilic drugs to hydrophilic metabolites prior to the drugs' excretion, and are considered of great significance in the overall biological handling of drugs.

A nomenclature system for P450 enzymes was implemented in 1987 based on the degree of similarity of primary amino acid sequences [5]. The members of separate gene families have ≤ 40% amino acid similarity (e.g. CYP1 vs. CYP2), while ≥ 59% similarity are assigned to the same gene subfamily (e.g. CYP2C). In other words, human loci are indicated by CYP followed by an Arabic number specifying the P450 family (e.g., CYP2), a letter for the subfamily (e.g., CYP2C) and an Arabic numeral for the gene, for example CYP2C19. The nomenclature system, however, depends neither on the function of P450 enzyme nor on the reactions they catalyze.

Of more than 100 CYP450 isozymes, only a dozen are responsible for the metabolism of the majority of drugs. CYP3A4/5 accounts for ~ 29% of the CYP450 content in human liver and 30% by reactions, based on data gathered from 248 clinically used drugs [6], [7] metabolized by the major CYP450 sub-families. The CYP2C subfamily accounts for ~18% by liver content and 24% by reactions, respectively, followed by CYP1A2 (~13% by liver content, 9% by reactions), CYP2E1 (7%, 3%), CYP2A6 (4 %, 3%), CYP2B6 (<1%, 7 %), and CYP2D6 (2%, 20%). The fractions of contribution of major isoforms in drug metabolism by reactions based on these 248 clinically used drugs are presented in Figure 1.

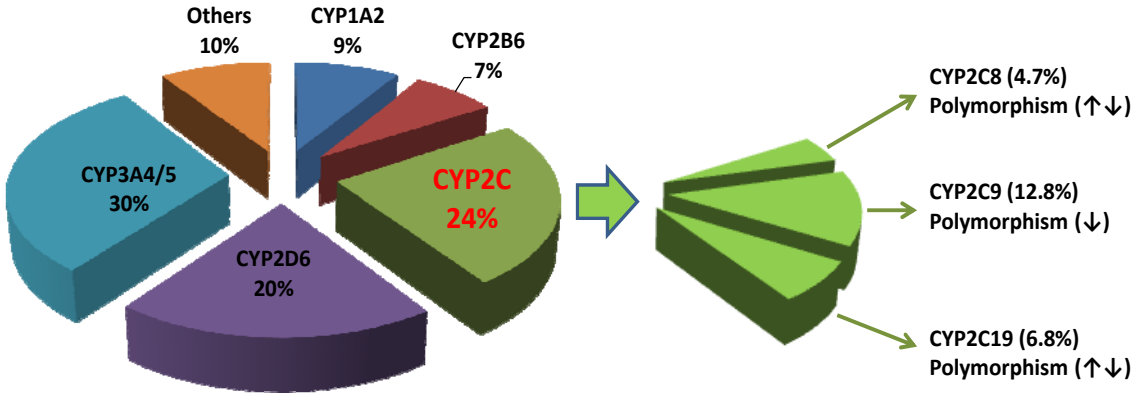

***Figure 1**. Fraction of contribution of CYP3A4/5, CYP1A2, CYP2B6, CYP2D6, CYP2C and further breakdown of CYP2C to CYP2C8, CYP2C9, and CYP2C19 in drug metabolism (based on 248 clinically used drugs).*

1.2.2 CYP2C subfamily

The CYP2C subfamily metabolizes approximately 20-25% of clinically used drugs. Four enzymes are identified in the CYP2C subfamily, namely CYP2C8, CYP2C9, CYP2C18, and CYP2C19. Of the subfamily CYP2C8, CYP2C9, and CYP2C19 are of clinical importance. The CYP2C enzymes generally have broad and overlapping substrate specificities. Some of them, however, demonstrated high activity against steroids, for example testosterone [8]. The CYP2C enzymes are also reported in developmentally regulated and sex-specific expression in animals [8], calling for cautions when using animal models to predict human responses. At the protein level, CYP2C9 is the highest expressed member in the 2C subfamily, comparable to CYP3A4 [9],[10]. The expression of CYP2C19 is 10-fold lower than that of CYP2C9. Despite the low expression level relative to CYP2C9, CYP2C19 remains an important polymorphically expressed CYP isoforms, participating in the metabolism of many widely prescribed drugs including omeprazole, citalopram, diazepam, and clopidogrel.

1.2.3 CYP2C19 enzyme

The CYP2C19 enzyme is a protein of 490 amino acids which binds to substrates that are generally neutral or weakly basic molecules. The drugs metabolized by CYP2C19 enzyme are classified as CYP2C19 substrates. Of these, the most commonly known substrates of CYP2C19 include proton pump inhibitors (PPIs: omeprazole, lansoprazole), certain antidepressants (citalopram/escitalopram, imipramine),

antiepileptics (diazepam, mephenytoin), the antimalarial drug proguanil, the β-adrenoceptor blocker propranolol and the antiplatelet drug clopidogrel.

CYP2C19 inhibition is observed with a number of molecules that bind to the enzyme and decrease the rate of substrate turnover. A few strong inhibitors of CYP2C19 are listed in FDA's guidance (fluconazole, fluvoxamine, ticlopidine), but none of them are CYP2C19 specific [4]. The mechanism of inhibition can be divided into reversible and irreversible inhibition (also known as time-dependent inhibition). Characterization of inhibitors are typically performed using *in vitro* methods [11], [12], [13] to generate IC_{50} or K_i. Prediction of likelihood of inhibition based on *in vitro* IC_{50} or K_i sometimes can be limited because the *in vitro* human tissues including hepatocytes do not represent true human physiological conditions. The clinical assessment of inhibition is, therefore, the most powerful approach, especially when studies are performed within the clinical relevance context. To study CPY2C19 inhibition, omeprazole can be used as a diagnostic probe in humans. Although it is a moderate inhibitor as classified by FDA, omeprazole has been proved to be a reliable and sensitive probe of CYP2C19.

The CYP2C19 enzyme is also inducible. If enzyme activity or the total amount of enzyme is increased, an elevation of intrinsic metabolic clearance is anticipated. From a PK perspective, the victim of enzyme induction is associated with reduced AUC, Cmax, Cmin (trough concentration) and elimination half-life. The mechanism of induction involves nuclear hormone receptors, i.e. constitutive androstane receptor (CAR) [14], pregnane X receptor (PXR) [15], and estrogen response element (ESE) [16], [17]. Rifampicin, a potent inducer of CYP2C19 in humans, has been reported as a classic ligand to PXR, initiating transcription of mRNA and production of active enzyme. The induction of enzyme activity is concentration-dependent and less than the induction of transcription mRNA level [18], [19]. Compared to CYP3A4, the induction of CYP2C19 activity is moderate. However, if a drug is metabolized by CYP2C19 and CYP3A4, both are induced by PXR (rifampicin), and the effect of induction can be additive, leading to a clinically significant interaction.

1.3 CYP2C19 POLYMORPHISM

Polymorphism has been employed to describe a trait that appears in a population as two or more phenotypes. It was defined by Vogel and Motulsky [20]: "A polymorphism is a

Mendelian or monogenic trait that exists in the population in at least two phenotypes (and presumable at least two genotypes), neither of which is rare - that is neither of which occurs with a frequency of less than 1-2%."

1.3.1 History of CYP2C19 polymorphism

The polymorphism of CYP2C19 is also known as "S-mephenytoin polymorphism" because CYP2C19 metabolizes S-mephenytoin to 4'-OH-mephenytoin. In the late 1940s, mephenytoin was introduced onto the market as an anticonvulsant agent administered as a 1:1 racemic mixture of the S - and R-enantiomers. In the 1970s, Adrian Küpfer, a Ph.D. student at the University of Berne, worked on an enantiomer project as part of his thesis and discovered the stereo-selective metabolism of mephenytoin in dogs [21]. Later, druing his postdoctorate training at Vanderbilt University, Küpfer and coworkers found the steroselectivity of S-mephenytoin metabolism in human subjects [22]. In this human study, one of the healthy volunteers complained of severe sedation after receiving a low dose of mephenytoin that had no effect on other individuals. Tracking the radio-labeled pseudo-racemic mephenytoin in urine samples, Küpfer *et al*. noticed a pronounced reduction of the 4'-OH-mephenytoin formed in this subject relative to others, suspecting polymorphism of drug hydroxylation for mephenytoin [23], [24]. A family study followed revealing a genetic basis for the impaired capability of 4'-OH-mephenytoin formation [25], [21]. Inaba and coworkers recruited five poor metabolisers and 28 relatives of the five PM proband, and clearly demonstrated that the pattern of deficient mephenytoin hydroxylation was an autosomal recessive trait [26].

The findings of the polymorphism and its genetic basis powered extensive research in the field. At the beginning, CYP2C9 was thought to be the major enzyme responsible for the formation of 4'-OH-mephenytoin. However, cDNA expressions showed negative results on all of the allelic variants of CYP2C9 toward S-mephenytoin, including a sample from the "mythical poor metaboliser" [27], [28], [29], [30]. The first positive sign was observed in purified protein by Wrighton *et al* [31], discovering an association between the hepatic content of this protein and the activity of S-mephenytoin hydroxylase. In 1994, Wrighton's group and Goldstein's group independently identified that CYP2C19 was primarily responsible for mephenytoin 4'-hydroxylation using immunoblot analysis [32] and yeast cDNA expression system [30], respectively.

1.3.2 Phenotyping of CYP2C19

CYP2C19 is one of the most studied drug-metabolizing enzymes that are polymorphically expressed in humans. When a polymorphism is involved in drug metabolism, individuals can be classified as either extensive metaboliser (EM) or poor metaboliser (PM) of a certain drug. In the case of CYP2C19, PM refers to the individual who lacks CYP2C19 enzyme activity based on phenotyping results.

Phenotyping methods comprise administration of probe drugs and quantization of the drugs and metabolites in plasma or urine to determine enzyme activity in the populations tested. A great deal of effort has been made to find proper phenotyping methods which accurately characterize the enzyme function. A desirable probe and testing procedure include the following: 1) safe without side effects at the dose used; 2) mainly metabolized by the enzyme that is phenotyped; 3) available on the market; 4) easily applicable in operation including a simple and robust analytical assay; 5) relatively inexpensive. With respect to CYP2C19 phenotyping, the mephenytoin S/R ratio in urine collected during the 8 hours post dose (if possible, extend to 8-24 and 24-32 hours collection) has been used for many years [33], [34]. Attributing to the desirable substrate specificity and a good understanding of drug characterization, the mephenytoin method was well accepted as a golden standard. The utility is, however, somewhat limited due to its low availability in markets [35].

Omeprazole has been intensively evaluated as a probe for CYP2C19 phenotyping including the work as part of this thesis. The Metabolic Ratio (MR) between omeprazole and 5-hydroxyomeprazole was validated by comparing to mephenytoin S/R ratio. The assay was widely adopted by users to identify the EMs and PMs in their studies [36]. As one of the five probes, omeprazole was selected in the Karolinska cocktail for phenotyping the five human CYP450 enzymes [37]. FDA now also recommends omeprazole to be used for evaluation of CYP2C19 metabolism both *in vitro* and *in vivo*. Other efforts towards phenotyping CYP2C19, such as using clobazam or lansoprazole, are also reported [38].

It is generally believed that phenotype expresses the current activity of the particular enzyme when the test is performed, and also reflects precise information about an individual's metabolism. Unlike inherited characteristics, the phenotype determined under certain circumstance may vary for the same individual as CYP450s are

susceptible to variability through induction and inhibition. A strong inhibitor or liver diseases could transfer an EM to a PM category [39], [40].

1.3.3 Interindividual variability

Polymorphism of drug metabolism enzymes is one of the major causes of high interindividual variability in the PK of CYP2C19 substrates. The variability often leads to marked differences in extent of metabolism, drug concentration-time profile or drug exposure and, consequently, pharmacological effect or toxicity.

The clinical impact of CYP2C19 polymorphism is well documented [41], [42], [43]. The best example on drug metabolism is the case of omeprazole [44]. The formation of hydroxyomeprazole is primarily mediated by CYP2C19 and polymorphic CYP2C19 activity has significant impact on the PK of omeprazole. When a single dose of 20 mg omeprazole was given to a group of EMs, IMs, and PMs, plasma concentrations of omeprazole differed remarkably among these three phenotypes in Japanese subjects. The relative AUC (area under curve) values in EMs, IMs, and PMs were 1:3.3:12.1 (421:1402:5108 ng•h/mL) [44]. A similar result was also observed with esomeprazole, the (S)-isomer of omeprazole [45]. For other PPIs, the exposure to lansoprazole or pantoprazole was found to be approximately 5-fold higher in PMs than in EMs of CYP2C19 [46].

Further influence on PK of PPIs is expected when coadministration of other drugs which are inhibitors or inducers of CYP2C19 are administered. Oral contraceptives (OCs) have been shown to inhibit the formation of hydroxyomeprazole, leading to more than 2-fold reduction of CYP2C19 activity in healthy EM subjects [47]. In PMs, the CYP3A4 mediated metabolic pathway of omeprazole to omeprazole sulphone became dominant due to impaired CYP2C19 metabolism. It is conceivable that inhibition of CYP3A4 in PMs could significantly change the PK of PPIs. Ketoconazole (200 mg/day), a strong inhibitor of CYP3A4, doubled the inhibition of omeprazole sulphone formation in PMs compared to EMs. For parent drug, the AUC of omeprazole increased 1.4-fold and 1.9-fold in EMs and PMs, respectively, after coadministration of ketoconazole [48].

1.3.4 Interethnic variability

The frequency of *CYP2C19* polymorphism shows marked interethnic differences. Numerous studies have been performed to determine the phenotype trait and frequency of PMs in various populations.

The distribution of PMs in selected ethnic groups is summarized in table 1. As presented, the frequency of CYP2C19 PMs is relatively high in Japanese [49], Chinese and Koreans [50], but low in Caucasians, Africans [51], [52] and Arab populations [53]. The presence of PMs were too low to be detected in Cuna Indians of Panama [54] whereas 79% of the population is PM on the island of Vanuatu in the Pacific Ocean [55], [56]. *CYP2C19*2*, **3* and their frequency, presented in Table 1, will be discussed in section 1.4.

Table 1. Distribution of PM and allele frequency of *CYP2C19*2* and **3* in selected ethnic groups

Ethnic group	**n**	**Allele frequency**		**PM%**	**Reference**
		*2	*3		
Caucasian					
Danish	358, 239	0.038	0	2.5	[57], [58]
Dutch	765, 4301	0.133	0.002	1.8	[59]
Italian	360	0.111	0	n.a.	[60]
Russian	290	0.114	0.003	2.3	[61]
Swedish	488, 253	0.15	0	3.3, 2.8	[62], [63]
African					
Ethiopian	114	0.136	0.018	5.3	[52]
Nigerian	92	n.a.	n.a.	4.3	[64]
Tanzanian	216, 251	0.10-0.179	0-0.03	4.6	[65], [66]
Zimbabwean	103	0.131	0	3.9	[67]
Middle East					
Saudi Arabian	97	0.15	0	2.1	[68]
Jewish Israeli	140	0.15	0.01	2.9	[53]
Indian and East Asians					
Indian (south, north)	341, 121	0.35, 0.30	0.01, 0	20.8	[69], [70]
Chinese (Han, Bai, Dai,Taiwanese)	101, 202,193, 118	0.26-0.37	0.034-0.075	13.4-19.8	[71], [72], [68]
Japanese	53, 140	0.23-0.35	0.104-0.11	15.1	[73]
Korean	103	0.21	0.012	12.6	[50]
Oceanian					
Faroese	312	0.029	0	0	[74]
Vanuatu	100, 5538	0.57, 0.633	0.25, 0.144	79	[56], [55]

1.4 GENETIC POLYMORPHISM OF CYP2C19

The CYP2C19 enzyme is encoded by the *CYP2C19* gene, which is located on chromosome 10q24 along with the other *CYP2C* genes in the order of *CYP2C18-CYP2C19-CYP2C9-CYP2C8* from centromere to telomer [7]. All members of the *CYP2C* sub-family exhibit genetic polymorphism.

Genetic variations in *CYP2C19* and molecular mechanism have been intensively studied using different methodologies with various materials. An initial study was performed in liver biopsy specimens from EMs and PMs, suggesting a deficiency of a specific CYP450 isozyme [75], [76]. Numerous efforts followed in several laboratories, including purification of proteins [77], [78], identification by antibodies [79], and expression of a CYP2C19 cDNA in yeast [30]. After tedious work with the highly homologous *CYP2C8, CYP2C9,* and *CYP2C19* genes, de Morais *et al* identified the first allele mutation and designated it *CYP2C19m1* (i.e. *CYP2C19*2*) [80], with reverse transcription and amplification of mRNA from liver samples. The second mutation was found in a DNA sample of a Japanese PM subject, defined as *CYP2C19m2* at the time (i.e. *CYP2C19*3*) [81]. When specifically searching for variants that could explain the large variability in enzyme activity within the EM phenotype and why some have such high activity, Sim and coworkers at Karolinska Institutet found a common novel CYP2C19 gene variant (*CYP2C19*17*) [82] that provides increased CYP2C19 activity by enhancing CYP2C19 expression.

1.4.1 Clinically relevant allele variants

To date, at least 34 allele mutants of *CYP2C19* have been documented at http://www.cypalleles.ki.se [83], an online CYP-allele database widely used around the world. Although most of the mutants are rare and functionally silent, the alleles *CYP2C19*2-*8* lead to either loss-of-function or reduced enzyme activity, explaining almost all PMs of CYP2C19. On the other hand, *CYP2C19*17* is associated with increased CYP2C19 activity and is also known as the "gain-of-function" allele. The wild-type *CYP2C19* allele, producing a normal function of CYP2C19 enzyme activity, is designated as *CYP2C19*1*. Table 2 lists dbSNP and main changes in cDNA and genes for the most common alleles that have clinical relevance.

Table 2* The common CYP2C19 polymorphisms structure and sites of change

Allele	Nucleotide changes		dbSNP	Trivial name	Enzyme activity
	cDNA	Gene			
CYP2C19*1					Normal
CYP2C19*2	681G>A	19154G>A	rs4244285	m1	None
CYP2C19*3	636G>A	17948G>A	rs4986893	m2	None
CYP2C19*4	1A>G	1A>G	rs28399504	m3	None
CYP2C19*5	1297C>T	90033C>T	rs56337013	m4	None
CYP2C19*6	395G>A	19294T>A	rs72552267	m5	None
CYP2C19*7		12748G>A	rs72558186		None
CYP2C19*8	358T>C	12711T>C	rs41291556		Reduced
CYP2C19*17		–806C>T	rs12248560		Increased

**based on http://www.cypalleles.ki.se [83]*

CYP2C19*2 variant is the result of a single base pair alteration G> A at base 681 located in exon 5, producing a premature stop codon 20 amino acids downstream. This change results in a truncated non-functional protein product [80].

CYP2C19*3 gene has a mutation 636G>A in exon 4, creating a premature stop codon and producing a truncated inactive enzyme [81].

CYP2C19*4 is a mutation of A>G at the first base of exon 1. This change alters the initiation codon, producing no protein product [84].

CYP2C19*5 is characterized by 1297C>T mutation in exon 9, leading to amino acid substitution $Arg^{433}Trp$ and markedly reducing enzyme activity [85].

CYP2C19*6 has a mutation 395G>A in exon 3, leading to amino acid substitution $Arg^{132}Gln$ and affecting structure and stability. This change results in an inactive enzyme [86].

CYP2C19*7 mutation is a result of T>A inversion at 5'-splice site, creating intron 5 splicing defect and affecting protein syntheses [87].

CYP2C19*8 is characterized by 358T>C mutation in exon 3, leading to amino acid substitution $Trp^{120}Arg$ and subsequently producing reduced enzyme activity [87].

CYP2C19*17 gene has 2 linked mutations, -806C>T and -3402C>T with high linkage in the 5'-regulatory region, leading to increased expression and activity [82].

1.4.2 *CYP2C19* genotyping and allele frequency

Although genomic DNA can be prepared from other sources, preparation of DNA from peripheral blood has been used in many studies for *CYP2C19* genotyping. PCR-based tests for the two most frequent loss-of-function alleles (*CYP2C19*2* and *CYP2C19*3*) were available soon after the mutations were reported. Attributing to the new technology applied in genomics, CYP genotyping has become robust, high-throughput and commercialized with standard procedures of validation and quality control (QC).

The frequency of *CYP2C19*2* allele was as presented in Table 1: ***0.038-0.15*** in Caucasians [88], [61], [89], [90], [91], [92], ***0.21-0.37*** in Indians and East Asians [50], [68], [69], [70], [71], [72], and ***0.10-0.18*** in Africans [51], [66], [67], [93]. The highest (***0.57-0.63***) and lowest (***0.029***) prevalence of the *CYP2C19*2* allele have been reported respectively from the island populations of Vanuatu [56] and Faroese [74]. With respect to *CYP2C19*3* allele, Vanuatu also showed the highest (***0.144-0.25***) frequency, following by Korean (***0.12***) [50] and Japanese (***0.104-0.11***) population [73], [94]. All Caucasians and Africans have the frequency of *CYP2C19*3* less than ***0.007*** (0.7%) except for Ethiopians who have a frequency of ***0.02*** (2%) [52]. For additional null alleles (*CYP2C19*4-*8)*, the inter-ethnic distributions are not available, perhaps due to extremely low incidence of each allele.

The higher frequency of mutant alleles, the larger portion of heterozygotes based on the Hardy-Weinberg equation. For enzyme activity, homozygous carriers (two identical copies) of functional *CYP2C19* genes differ from heterozygous carriers (one copy), following the gene-dose-effect pattern. For example, heterozygous for *CYP2C19*2* or **3* (i.e. *CYP2C19*1/*2* or *CYP2C19*1/*3* genotype, also known as heterozygous EM) is associated with lower enzyme activity as compared to carriers of **1/*1* (homozygous EM). In Asians, the portion of heterozygous EMs is about twice as high as in Caucasians. A higher AUC of omeprazole was seen in Chinese EMs (2.6 μM•h) than Caucasian EMs (0.9 μM•h) with comparable AUCs in PMs (13.3 in Chinese vs. 11.1 in Caucasians), reflecting that Chinese EMs have lower CYP2C19 activity [95]. The higher portion of heterozygous EMs in Chinese is also likely the main reason to explain the slower clearance of diazepam in this population compared to Caucasians [96].

The frequency of *CYP2C19*17* allele was reported to be ***0.18*** in Caucasians and ***0.04*** in Asians. This gain-of-function allele is, however, less studied in other ethnic groups compared to the loss-of-function alleles because it was undiscovered until 2006 [82].

1.4.3 Nongenetic host factors influencing CYP2C19 expression and function

There are still high inter-individual and inter-ethnic differences in the metabolism of CYP2C19 substrates within homozygous carriers of *CYP2C19*1*. Heritable genetic variation cannot explain all differences affecting CYP2C19 expression and function. As reported in some studies [97], [98], ***age*** contributes to reduction of drug clearances and long half-life. The capability of drug metabolism appears substantially lower (29-45%) in the elderly population (70-100 years) compared with the young control group (20-50 years) [99]. However, no significant differences are found in CYP enzyme activity between young and old populations [100]. It is generally believed that decline of liver blood flow and liver volume with age in humans is part of reasons responsible for the diminished clearance of drugs in elderly population. The effect of ***gender*** on the activity of CYP2C19 is controversial. Both higher or lower activity in females than males have been reported [94], [101]. Generally, sex affects PK through body weight, fat distribution, liver blood flow, and expression of enzymes and transporters, but no gender bias was found in the expression of CYP2C19 in a gene expression profiling study where 40 ADME-related genes were screened in 112 male and 112 female livers [102]. Oral contraceptives (OC) are proven to be moderate inhibitors of CYP2C19 [47]. As OC are often not considered as an exclusionary criterion in clinical trials, the PK results can be confounding for the women with child-bearing potential who take OCs regularly.

Disease states also influence enzyme activity. For CYP2C19, it has been documented to be moderately reduced in liver disease [103], [104]. This is perhaps in part due to one or more of the following reasons: 1) reduction of liver blood flow; 2) loss of functional hepatocytes; 3) alternation of the architecture of the liver; and 4) lower synthesis of serum proteins.

Epigenetic factors also potentially influence CYP2C19 metabolism, for example miRNAs regulating gene expression. Of special interest for pharmacogenetic aspects are SNPs in miRNAs and miRNAs binding site as well as miRNAs copy number

variations [105]. Although there are no direct examples showing an impact of miRNAs on CYP2C19, the results of CYP2C8 being regulated post-transcriptionally by miR-103 and miR-107 [106] demonstrates an influence of miRNAs on ADME regulation and potential relevance in drug response.

1.5 CLINICAL IMPACT OF CYP2C19 POLYMORPHISM

Individuals vary greatly in their response to drug therapy. After receiving a standard dose of a drug that is recommended based on a population average, some patients could have insufficient response, whereas others may experience adverse effects. Because CYP2C19 plays an important role in metabolizing several widely prescribed drugs, such as omeprazole, citalopram, and clopidogrel, the clinical relevance of genetic polymorphism to the efficacy and safety of drugs has received a lot of attention. Although all drugs metabolized by CYP2C19 are likely to show clinical importance on genetic polymorphism, the drugs for which CYP2C19 is the primary metabolic pathway and those with narrow therapeutic ranges are particularly susceptible.

1.5.1 Variability in drug response

Both PK and PD determine intensity and duration of a pharmacologic agent. After a drug is administered, it will undergo ADME/ PK processes while interacting with the PD target. For drugs that are CYP2C19 substrates, the enzyme polymorphism is deemed to be one of the independent determinants among a number of factors that contribute to the inter-individual variability.

The influence of CYP2C19 polymorphism on omeprazole PK has been discussed in section 1.3.3 discussing interindividual variability. The effect of CYP2C19 polymorphism on PD is also primarily studied with omeprazole. Furuta *et al* investigated intragastric pH in 16 healthy subjects following administration of omeprazole [44]. Significant differences were observed among the three phenotype groups, and the mean values of 24-hr intragastric pH in EMs, IMs, and PMs were 2.1, 3.3 and 4.5, respectively. The first study in this thesis also demonstrated that omeprazole increases plasma gastrin more in PMs than that in IMs or EMs, and the level of increase is correlated with the plasma AUC of omeprazole. In addition to the intragastric pH and plasma gastrin, several other PD parameters including pepsinogen I, plasma chromogranin A, and oxyntic

mucosa were reported to be affected in a manner consistent with CYP2C19 polymorphism [107], [108].

Clopidogrel is a clinically important cardiovascular drug that requires oxidation to form its active thiol metabolite. As presented in Figure 2, CYP2C19 participates in every step of activation among multiple CYP enzymes, so its role in the conversion of clopidogrel is clearly demonstrated.

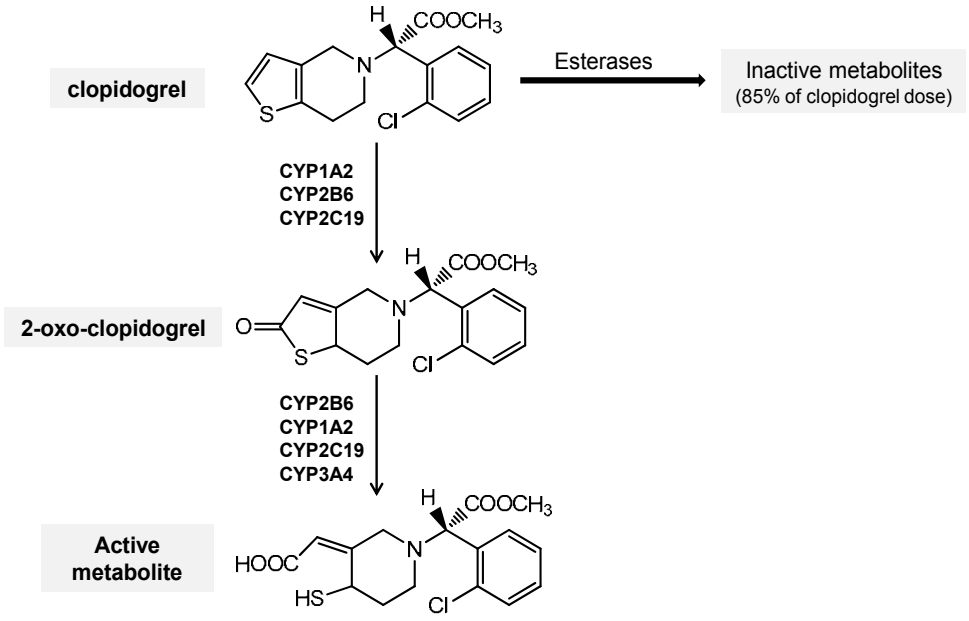

Figure 2 *Structure of clopidogrel, metabolites, and metabolic pathways*

There is large inter-individual variability in clopidogrel response. Depending on the criteria used, approximately 15-40% are considered non-responders with high residual platelet aggregation [109], i.e. "clopidogrel-resistant". Genetic variation in the *CYP2C19* gene leads to significant differences in exposure to the active metabolite, and subsequently translates the change into clinically meaningful impact on cardiovascular events (e.g., death, stent thrombosis, myocardial infarction, and stroke) up to one year follow-up periods. For *CYP2C19* poor metabolisers, high residual platelet reactivity with a 3-fold increase in one year incidence of death and myocardial infarction has been observed [110]. These findings of CYP2C19 PMs with significantly lower anti-coagulation effect of clopidogrel and higher risk of major adverse cardiovascular events are supported by a number of clinical studies [111], [112], [113], [114], [115]. In 2009 and 2010, FDA modified the label of Plavix (clopidogrel) by adding warnings in its package insert. The agency also alerted patients and clinicians [116] that the drug can be

less effective in people who have reduced CYP2C19 function, for example PMs. In order to manage the risk, FDA also recommends *CYP2C19* genotyping be considered prior to prescribing the drug.

1.5.2 Application of pharmacogenetics and personalized medicine

As discussed in clopidogrel case, public interest in pharmacogenetics has increased specifically during the human genome project and since its completion on 4/4/2013. The terms of "pharmacogenomics", "pharmacogenetics", "genomic biomarker", and "personalized medicine**"** often appeared in reports/publications with slightly different definitions. To setup a scope of discussion, the terms used in this thesis are defined as follows:

- ***Pharmacogenomics*** - The study of variations of DNA and RNA characteristics as related to drug response
- ***Pharmacogenetics*** - The study of variation in DNA sequence as related to drug response.
- ***Genomic biomarker*** - a measurable DNA and/or RNA characteristic that is an indicator of a normal biological or a pathogenic process and/or a response to therapeutic or other interventions
- ***Personalized medicine*** -The application of genomic and molecular data to improve the delivery of healthcare, facilitate the discovery and clinical testing of new products, and help determine individual predisposition to a particular disease or condition.

The first three definitions are adapted from Note for Guidance on Definitions for Genomic Biomarker, Pharmacogenomics, Pharmacogenetics, Genomic Data and Sample Categories (EMEA/CCHMP/ICH/437986/ 2006) and the last one is adapted from the US Genomics and Personalized Medicines Act 2007.

The information generated from genotyping/phenotyping tests can be tailored to guide drug choice and/or dosage regimen for an individual patient. Pharmacogenetics-guided warfarin dosing algorithms provide an example of personalized medicine using demographic, clinical, and genetic factors. Warfarin is known to have more than a 10-fold individual variation in dosage requirement. Genetic polymorphism of the pharmacological target gene (*VKOR*) and metabolism gene (*CYP2C9* encode enzyme

CYP2C9 for metabolizing S- warfarin) contribute to the large interindividual variability of warfarin. Together with age and bodyweight, variation in genes account for more than 50% of variance in dosage requirement. Unlike the standard practice of "one-dose-fits-all", a number of algorithms by incorporating genetic, clinical factors, age and body size have been proposed for maintenance dosing of warfarin [117], [118], [119]. Using internet-based clinical trial protocols [120], the Swedish WARG study (The Warfarin Genetics) had a large sample size adequately powered to test smaller effects among 29 candidate genes. In 1496 Swedish patients who started warfarin treatment, 183 polymorphisms in the 29 genes were genotyped [121], and 59% of the variance in warfarin dosing could be explained by *CYP2C9*, *VKORC1*, age, sex and drug interactions.

The approach of personalized medicine is not always as smooth as expected. We discussed the cases of clopidogrel in Section 1.5.1 where the clinical utility of genotyping was clearly demonstrated in a number of studies including a large trial [111] consisting of 162 healthy subjects and 1477 patients. This TRITON-TIMI study published in New England Journal of Medicine, revealing 53% relative increase in the risk of death from cardiovascular causes or stroke for carriers of at least one loss-of-function allele of *CYP2C19* as compared with non-carriers. However, a number of studies, including two meta-analyses [122], [123], have recently concluded that the predictive value of pharmacogenetic testing prior to clopidogrel treatment is limited. The results deviated largely from FDA's recommendations. The American Heart Association and American College of Cardiologists also challenge whether the evidence is sufficient to support the genotype-guided prescription paradigm.

1.6 CYP2C19 SUBSTRATES

The following are the typical CYP2C19 substrates related to this thesis:

1.6.1 Omeprazole

Omeprazole was the first proton pump inhibitor in clinical practice. It effectively suppresses gastric acid secretion and is widely used to treat acid-related disease [124]. The drug acts by irreversible binding to the H^+-K^+ ATPase in the parietal cell [125] and the anti-secretory effect is related to the area under the plasma concentration-time curve (AUC) of omeprazole. PK of omeprazole has been well documented in both healthy and duodenal ulcer patients [126], [127]. With respect to elimination, omeprazole is nearly completely metabolized in the liver, mainly by CYP450 enzymes. As presented in Figure

3, CYP2C19 is responsible for the metabolism of omeprazole to 5-hydroxyomeprazole and omeprazole sulphone to 5-hydroxyomeprazole sulphone while CYP3A4 is involved in the sulphoxidation pathways.

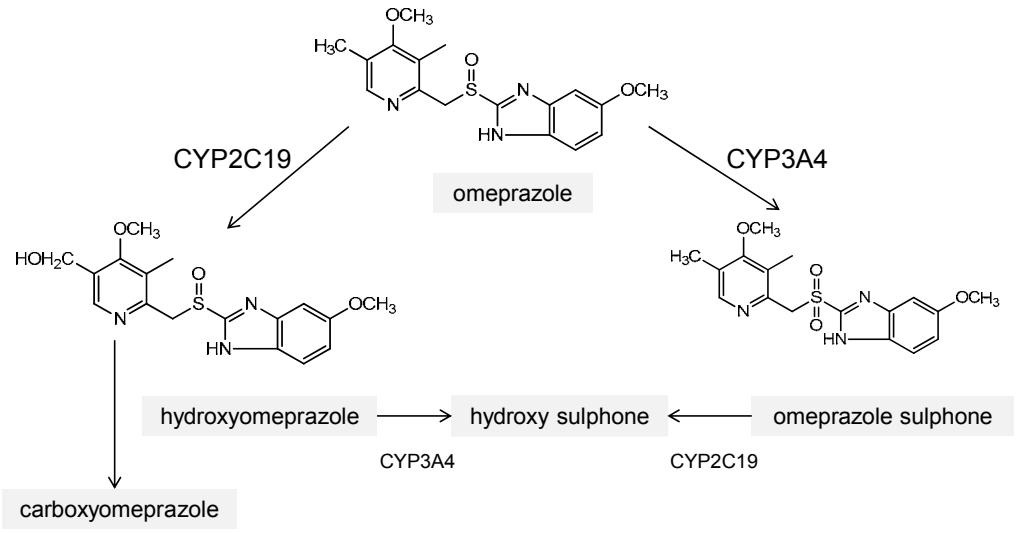

Figure 3 *Structure of omeprazole and metabolites*

Kinetic parameters for omeprazole to 5-hydroxylation activities were determined by recombinant CYP2C19 in insect microsomes. The low *Km* and high *Vmax* values generated from *in vitro* methods indicated that omeprazole is an efficient substrate for CYP2C19 [128]. Moreover, the role of CYP2C19 enzyme in the metabolism of omeprazole has been well characterized in a number of clinical studies including healthy subjects and patients. Based on the robust enzyme-substrate characterizations, omeprazole was further selected as a probe drug in clinical evaluation to help understand the nature and magnitude of DDIs where CYP2C19 may be involved [4], [37].

1.6.2 Citalopram/escitalopram

Citalopram and escitalopram are selective inhibitors of serotonin reuptake (SSRI) widely used for the treatment of depression and anxiety disorders [129]. Escitalopram is the S-enantiomer of racemic citalopram and binds with high affinity to the human serotonin transporter [130]. Overall, approximately 85% of the drug(s) is eliminated by the liver and the rest by the kidney. CYP2C19 is the major enzyme in the liver catalyzing the metabolism of citalopram followed by CYP3A4 and CYP2D6 to a lesser extent [131] (Fig 4). The role of CYP2C19 in N-demethylation of citalopram was demonstrated in cDNA expressed human cytochrome. The metabolite

desmethylcitalopram (DCT) is significantly less active and its contribution to the overall action of citalopram is negligible. Compared to young and healthy subjects, the elderly and patients with hepatic or renal failure have slower elimination processes. Potent inhibitors of CYP2C19 and 3A4 might decrease citalopram clearance [132].

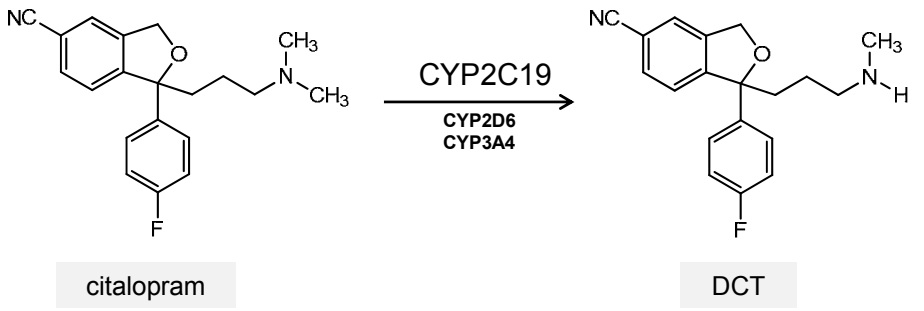

Figure 4 *Structure of citalopram and its demethylation metabolite (DCT)*

Since *CYP2C19*2* and *CYP2C19*3* account for the majority of loss of function alleles associated with the PM phenotype, several studies have investigated the effect of *CYP2C19*2* and *CYP2C19*3* on citalopram exposure and its clinical implications. For *CYP2C19*17,* a few studies have been conducted to examine the impact of this gain-of-function allele on steady state citalopram concentrations and clinical outcomes [133], [134], [135].

The pharmacogenetic studies of citalopram have yielded somewhat inconsistent results, especially with regard to the quantitative impact of the *CYP2C19*17* allele. Most studies were not powered to provide useful quantitative estimates of the genotype effect. From a PD standpoint, fewer than 50% of patients treated with SSRI including citalopram experience a complete remission of their symptoms [136]. Among a number of factors contributing to the low response rate, insufficient drug exposure is deemed to be one of the reasons.

1.6.3 Warfarin

Warfarin is an oral anticoagulant, commonly described as vitamin K antagonist and used for the treatment and prevention of thromboembolic disorders [117]. Its anticoagulant effect is mediated via inhibition of the enzyme vitamin K epoxide reductase (VKOR) [137]. Due to a narrow therapeutic index and large interindividual variability in dose requirement, the clinical use of warfarin is challenging. The effectiveness and safety of

warfarin is dependent on administering an individually titrated dose to maintain prothrombin time, expressed as the international normalized ratio (INR or PT-INR) within a therapeutic range of 2.0-3.0.

Warfarin is administered as a racemic (1:1) mixture of its S- and R-enantiomers. Both the PK and PD of S- and R-warfarin differ. S-warfarin is considered to be 3-5 times more potent than R-warfarin as an inhibitor of VKOR. S-warfarin is almost exclusively metabolized by the cytochrome P450 CYP2C9 [138]. The metabolism of R-warfarin, on the other hand, is catalyzed by multiple CYP enzymes including CYP2C19, CYP3A4, CYP1A2, and possibly CYP2C8 (Fig 5), as assessed *in vitro* using recombinant CYP2C19, human liver microsomes and chemical inhibitors by Kim So-Young *et al* [139].

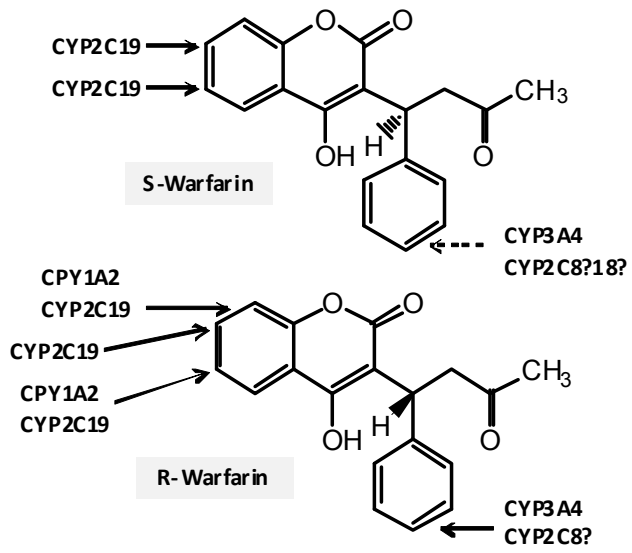

Figure 5 *Structure of S-, R-warfarin and metabolic pathways*

CYP2C9 polymorphisms have been well documented as a major determinant on S- (but not R-) warfarin clearance, and on warfarin dose requirement. Compared to S-warfarin, the PK and PD of R-warfarin are much less studied. In a single dose study in Japanese healthy volunteers, an approximately 30% higher AUC of R-warfarin was demonstrated in CYP2C19 poor metabolisers compared to extensive metabolisers [140]. None of the studies published so far have addressed the potential impact of the *CYP2C19*17* allele, associated with increased CYP2C19 activity and contributing to variability in enzyme activity within the extensive metaboliser group, on warfarin clearance and dose requirement.

2 AIM

The overall aim of this thesis was to investigate the influence of CYP2C19 polymorphisms on PK and PD of clinically important CYP2C19 substrates, to quantify the effect of functional *CYP2C19* allele variants including the gain-of-function allele (*CYP2C19*17*) on drug exposure and response, to advance knowledge and understanding of inter-individual variability in drug therapy. The specific aims of the individual studies were the following:

Study I: To investigate the disposition of omeprazole and its effect on plasma gastrin levels after single and multiple oral doses in the three phenotype groups (PMs, IMs, and EMs) of S-mephenytoin hydroxylation.

Study II: To investigate the distribution of the ratio between the 3-hour plasma concentrations of omeprazole and hydroxyomeprazole (i.e. metabolic ratio, MR) in a large healthy Swedish population and to assess the correlation between the MR of omeprazole and the S/R ratio of mephenytoin as well as the CYP2C19 genotype.

Study III: To assess the potential for pharmacokinetic interactions between carbamazepine (CBZ) and omeprazole and to study the inducibility of CYP3A4 and CYP2C19 by CBZ, using omeprazole as a probe for these two enzyme activities.

Study IV: To assess the potential influence of *CYP2C19*17* on R-warfarin clearance, and the combined effect of *CYP2C19*, *CYP2C9* and *VKORC1* polymorphisms together with non-genetic factors on warfarin dose and INR response. The S/R ratio of warfarin in relation to *VKORC1* genotype was also investigated.

Study V: To quantify the effect of functional CYP2C19 allele variants on citalopram/escitalopram exposure by means of systematic review and meta-analysis.

3 METHODS

3.1 SUBJECTS

Interphenotype study (I)

Fourteen (14) previously phenotyped healthy Swedish volunteers were selected and divided into three groups with respect to the S/R ratio of mephenytoin. Five (4 males and 1 female) with an S/R ratio close to 1 (0.91-1.10) were PM. Another 5 subjects (4 males and 1 female) were rapidEM with an S/R ratio < 0.05. The remaining 4 (3 males and 1 female) volunteers had an S/R ratio range between 0.27 and 0.75, and were classified as heterozygous EM (hetEM) because each of them has a PM parent. All subjects were between 23 and 33 years of age.

Population study (II)

One hundred and sixty (160) healthy Swedish Caucasian subjects (72 males and 88 females) were recruited. The subjects were between 19 and 54 years old. Most were from the staff of the Huddinge Hospital, medical students at Karolinska Institute, and their friends. Subjects who had no drugs, except for oral contraceptives, one week before or during the study were eligible to the study.

Carbamazepine study (III)

Five Swedish subjects (4 females and 1 male) with newly diagnosed epilepsy requiring long term treatment of carbamazepine participated. The patients were between 23 to 66 years of age.

Warfarin study (IV)

This study included 150 (99 males and 51 females) Italian outpatients from two previously published clinical studies. The patients were between 22 to 87 years old and all were on stable maintenance doses of warfarin titrated to a target INR value between 2.0 and 3.0.

Meta-analysis study (V)

The studies where subjects received at least one single oral dose of citalopram or escitalopram and had been either phenotyped or genotyped with at least two *CYP2C19* alleles (**1, *2, *3, *17*) were included. All reports deemed eligible for inclusion were retrieved in full-text and the relevant data were re-extracted independently. No

restrictions were applied to subject characteristics, treatment duration, or concomitant drugs. A total of 987 subjects from 16 studies were included of which 39.6% were men. The age of the participants ranged from 15 to 84 years.

All clinical trials were approved by the Regional Ethical Review Board (or Committee). All studied were performed according to ICH-GCP guidelines, the declaration of Helsinki, and applicable local legislation.

3.2 ANALYTICAL METHOD

3.2.1 Genotyping

Population study (II)

DNA was isolated from peripheral leukocytes using a guanidinium isothiocyanate method. The defective *CYP2C19m1* allele was identified by PCR amplification of DNA with use of specific primers as described by de Morais *et al* [81]. After 35 cycles, the PCR products were digested with *Sam I* restriction enzyme overnight. Both the PCR products before and after digestion were analyzed on 3% agarose gels stained with ethidium bromide to determine the genotype of *CYP2C19* for *CYP2C19*2*.

Warfarin study (IV)

DNA was extracted from peripheral leukocytes using the Qiagen Cell Culture DNA kit. Five single nucleotide polymorphisms (SNPs) were genotyped using validated TaqMan genotyping assays from Applied Biosystems (for rs12248560C>T, *CYP2C19*17*; for rs4986893G>A, *CYP2C19*3*; for rs28399504A>G, *CYP2C19*4*; for rs10509681A>G, *CYP2C8*3*; for rs992323G>A, *VKORC1*2*). The analyses were carried out using an ABI PRISM 7500 Real-Time PCR System or an Applied Biosystems StepOnePlus Real-Time PCR system. The genotypes of *CYP2C19*2*, *CYP2C9*2* and *CYP2C9*3* were available since before as described [141].

3.2.2 Drug Analysis

3.2.2.1 Omeprazole and its metabolites

A liquid chromatography method was employed to quantify plasma concentration of omeprazole, hydroxyomeprazole, and omeprazole sulphone in ***Studies I, II,*** and ***III*** by

the HPLC method of Lagerström *et al* with modification [142]. Standard curves in the range of 0-500 nM were prepared on each day of analysis. The intra-day and inter-day variation (CV) for omeprazole and its metabolites were <10%.

3.2.2.2 Carbamazepine and its metabolites

Plasma concentration of carbamazepine and its 10, 11-epoxide metabolites were determined in ***Study III*** by HPLC according to a method developed by Tomson *et al* [143].

3.2.2.3 S-warfarin and R-warfarin

S-warfarin and *R*-warfarin plasma concentrations were determined in ***Study IV*** by an HPLC method developed by Henne *et al* [144] with modifications described by Scordo *et al* [141].

3.2.3 Gastrin

Plasma gastrin levels were analyzed in ***Study I*** by radioimmunoassay according to Nilsson [145] using antibody 4562 (generously supplied by Professor Jens Rehfeld, Denmark) and synthetic human gastrin I (Milab, Malmö, Sweden) as a tracer.

3.3 DATA ANALYSIS AND STATISTICAL METHOD

PK parameters were calculated using non-compartmental analysis (NCA) technique. Cmax was noted as the maximum concentration measured during the dosage interval. The area under the plasma concentration versus time curve (AUC) was estimated using the trapezoidal rule. The plasma elimination half-life ($T_{1/2}$) was obtained by least squares linear regression analysis of the terminal log plasma concentration vs. time curves. Assuming complete absorption of the drug in the gastrointestinal tract, the oral plasma clearance (CLo) was calculated by dividing the given dose by AUC.

Statistical software programs were employed to calculate significance of differences and correlations as described in the respective paper. PK parameters were log-transformed to reduce non-normality. Descriptive statistics included calculation of median and interquartile range or total range.

Methods for between-group comparison included the Mann-Whitney U-test (***papers I, II, IV***), unpaired Student's test (***paper I***), paired Student's test (***paper III***), and analysis

of variance (ANOVA) (***paper IV***) for continuous variables, and Pearson's Chi-square test (***paper IV***) for categorical variable. When the overall test showed a significant difference among the groups, Bonferroni test was used as *post hoc* analysis to elucidate the difference between individual groups (***paper IV***).

Regression analysis was performed by Spearman's rank correlation in the relations between mephenytoin S/R ratio and ratio of omeprazole over hydroxyomeprazole (***papers I*** and ***II***), and the relation between AUC of omeprazole and that of gastrin (***paper I***). Multivariate linear regression was used to assess the influence of continuous or categorical predictors on continuous outcome (***paper IV***), and the adjusted value of R^2 was a measure of the portion of variance.

Pooled effect estimates were calculated by means of fixed effect and random effects meta-analyses from several studies in ***paper V***. The individual studies were weighted according to inverse variance. The Cochran's Q test and the percentage of total variability across studies (I^2) were used to assess heterogeneity among studies. Potential sources of heterogeneity and bias from small study effects were analyzed by a univariate linear regression and funnel plots, respectively.

All tests were two-tailed, and p-values <0.05 were considered to be statistically significant.

4 RESULTS

4.1 PAPER I

4.1.1 Study design

Fourteen subjects were assigned to the groups of PM (N=5), rapidEM (N=5), and hetEM (N=4) based on the S/R ratio of mephenytoin. Omeprazole 20 mg was administered orally once daily for 8 days. Blood samples were drawn at predose and selected hours post dose. Omeprazole, hydroxyomeprazole, omeprazole sulphone and gastrin levels were determined. The PK parameters (Cmax, AUCs, CLo, and$T_{1/2}$) were derived.

4.1.2 Results

When 20 mg of omeprazole was given orally for 8 days, plasma concentrations differed markedly among the three different CYP2C19 phenotypes; rapidEMs (or EMs), hetEMs (or IMs) and PMs. The mean AUC values of omeprazole were 1130, 5984 and 14820 nM•h for EMs, IMs and PMs, respectively, with a relative ratio of 1:5.3:13.1 (Fig 6). Significant differences in the plasma gastrin levels (used as a PD marker in the study) were also observed among the three phenotype groups following the 8th dose. The mean AUC(0-10h) values of gastrin were 246, 555 and 621pM•h respectively, for EMs, IMs and PMs of CYP2C19, while the corresponding gastrin AUC values after the 1st dose were 221, 254 and 333 pM•h (Fig 7). There was a doubling of the AUC of gastrin after the eighth dose compared with the first omeprazole dose in both PM and IM groups.

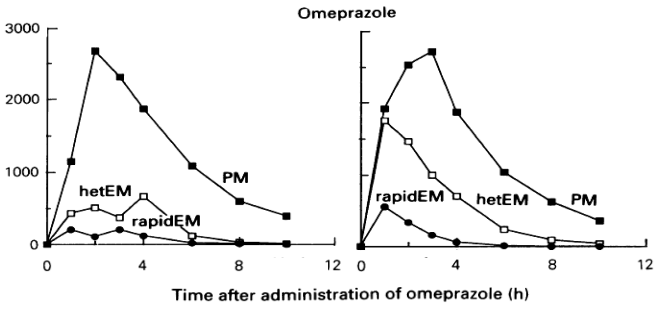

Figure 6 *Left panel: Mean plasma concentrations of omeprazole in rapidEM (● n=5), hetEM (□ n=4) and PM (■ n=5) of S-mephenytoin following the first dose of 20 mg omeprazole orally. Right panel: as in the left panel, but the mean plasma concentrations were measured after the eighth dose following administration of 20 mg omeprazole once daily.*

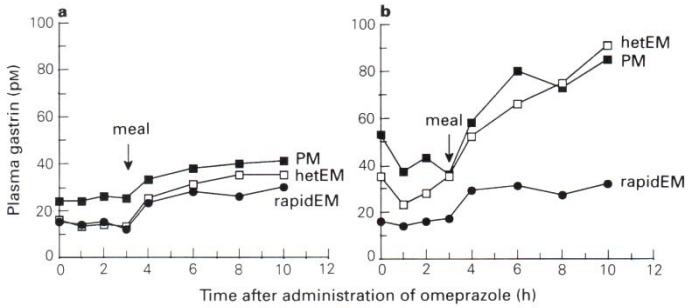

Figure 7 *a) Mean gastrin levels in the three groups following a single 20 mg omeprazole dose. There was no significant difference between the three groups either before or after drug administration. b) Mean gastrin levels after the eighth dose of omeprazole.*

4.2 PAPER II

4.2.1 Study design

One hundred and sixty healthy (160) subjects were phenotyped with mephenytoin one week or more after omeprazole dosing except for 21 subjects with a known mephenytoin phenotype. All subjects received a single 20 mg dose of omeprazole orally. Two blood samples were drawn at 3 hours post dose: one for omeprazole and hydroxyomeprazole quantification, and another for analysis of *CYP2C19*2*. The ratio of omeprazole to hydroxyomeprazole was calculated.

4.2.2 Results

A significant correlation was found between the metabolic ratio (MR) of omeprazole and the S/R mephenytoin ratio. The MR of omeprazole varied between 0.10 and 23.8 among the 160 subjects. Homozygous carriers of *CYP2C19*1* allele had lower MRs of omeprazole and S/R ratios of mephenytoin relative to heterozygous and homozygous carriers of *CYP2C19*2* allele. A good agreement was obtained between genotype and phenotype using either omeprazole or mephenytoin as a probe.

As presented in Fig 8, log MR of omeprazole (left panel) among EMs (wt/wt) was fairly normally distributed as compared to S/R ratio of mephenytoin (right panel) where 18% of the subjects had an S/R ratio of <0.05. Thereby the omeprazole assay might be capable to identify subjects with a very rapid MR of omeprazole or high CYP2C19 activity.

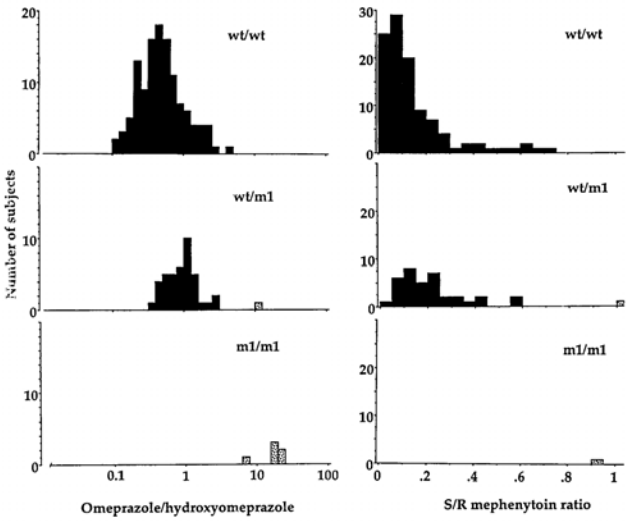

Figure 8 *Frequency distribution of the MR of omeprazole (left: n =160) and of the S/R mephenytoin ratio (right: n =141). Subjects showed as black bars were phenotyped as EM using mephenytoin as a probe drug; PMs were showed as shaded bars. Within the EM phenotype, the genotypes wt/wt and wt/m1 were different with respect to the omeprazole/hydroxyomeprazole ratio (wt/wt, n =113; wt/m1, n=40; p=0.0001) and S/R mephenytoin ratio (wt/wt, n =99; wt/m1, n=35; p=0.0001) using the Mann-Whitney test.*

4.3 PAPER III

4.3.1 Study design

Five patients requiring long term CBZ treatment were treated twice daily with a daily CBZ dose of either 400 mg (n=3) or 600 mg (n=2). Prior to starting CBZ treatment and after 3 weeks of CBZ treatment, the patients received a single oral dose of 20 mg omeprazole. Blood samples were collected at selected hours after omeprazole intake. Omeprazole, hydroxyomeprazole, omeprazole sulphone, CBZ, and the CBZ epoxide metabolite were determined. The PK (Cmax and AUC) of omeprazole and its metabolites before and during CBZ treatment were calculated. The AUC ratio of hydroxyomeprazole/sulphone was used to estimate the relative influence of CBZ treatment on the activity of the two CYP isoforms (CYP2C19 and CYP3A4).

4.3.2 Results

Both omeprazole and hydroxyomeprazole decreased by approximately 40% in mean AUC after coadministration of omeprazole with CBZ while the sulphone metabolite increased by 44% (Fig 9). None of the changes were statistically significant. The AUC ratios of omeprazole/hydroxyomeprazole were 0.85 and 0.75 with or without carbamazepine, suggesting little impact of carbamazepine on CYP2C19 activity. The AUC ratios of omeprazole/ omeprazole sulphone were 0.80 and 1.93 (58.5 % reduction, p=0.052) with or without carbamazepine intake. A significant decrease (2.58-0.93, *P*=0.046) was observed in the AUC ratios of hydroxyomeprazole/ sulphone metabolites, suggesting that the impact of carbamazepine was different on the two CYP isoforms

(CYP2C19 and CYP3A4) with more pronounced influence for CYP3A4 than for CYP2C19.

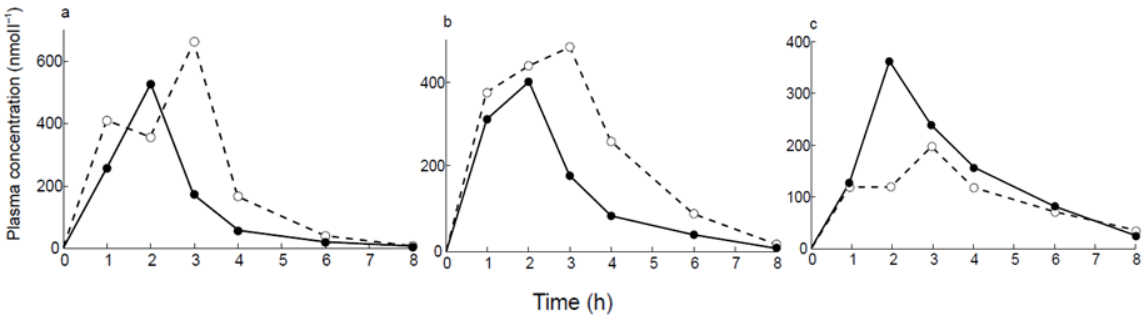

Figure 9 *Mean plasma concentrations of a) omeprazole; b) hydroxyomeprazole; and c) omeprazole sulphone in 5 patients given a single oral 20 mg dose of omeprazole before (○) and during (●) carbamazepine treatment.*

4.4 PAPER IV

4.4.1 Study design

This study was based on 150 Italian warfarin-treated patients (on stable maintenance dose of warfarin) included in two previously published studies, study A (N=93) [141] and study B (N=57) [146]. Plasma samples were taken at steady state (12-14 hours after the last dose) for determination of S- and R-warfarin concentrations and INR. The clearances of S- and R-warfarin were calculated. All patients were genotyped for *CYP2C19*2, *3, *4* and **17, CYP2C9*2* and **3, CYP2C8*3* and *VKORC1*2*, of which *CYP2C19*2*, *CYP2C9*2* and**3,* and partial *VKORC1*2* (study B) were available since before.

4.4.2 Results

As presented in Fig 10, *CYP2C19*17* carriers showed a 1.3-fold higher mean R-warfarin clearance (2.5 mL/min) than *CYP2C19*2* carriers (1.9 mL/min). Patients with *CYP2C19*1/*1* genotype had their mean R-warfarin CL in-between the **17* and **2* carrier groups (2.1 mL/min) and those who carried both *CYP2C19*2* and**17* alleles displayed a mean CL similar to that of the **17* carriers (2.5 mL/min). The increase in the latter 2 groups was not statistically significant. For the influence on INR/daily dose, *CYP2C19* genotype showed a significant difference (p=0.04) in addition to the *CYP2C9* (p<0.0001) and *VKORC1* (p<0.0001) genotypes. The contribution to the variability of INR/daily dose was 7%, 36%, and 27% for *CYP2C19*, *VKORC1*, and *CYP2C9* genotypes, respectively. Age also explained 7% of the variance.

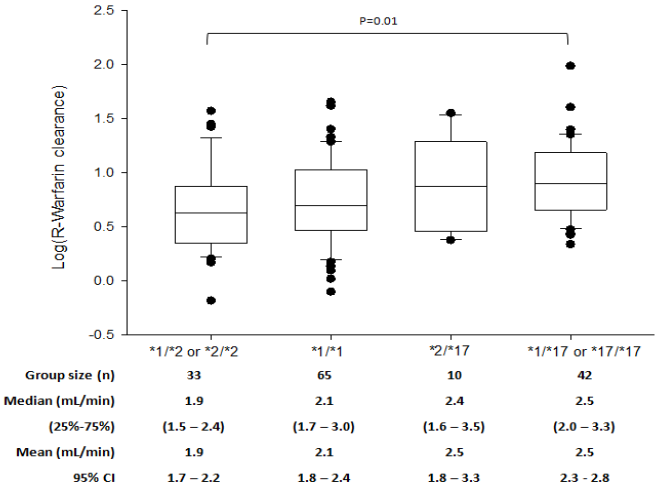

Figure 10. *R-warfarin clearance categorized by CYP2C19 genotypes. Log-transformed R-warfarin clearance with median and inter quartile are shown graphically and antilog values are presented numerically. Bonferroni post-hoc test with mean and 95% conference interval (CI) was used in multiple pairwise comparisons on the basis of log-transformed values. Statistical significant are showed between heterozygous and homozygous carriers of CYP2C19*2 and CYP2C19*17.*

4.5 PAPER V

4.5.1 Study design

Systematic review and meta-analysis were preformed to assess the impact of CYP2C19 genotypes on citalopram/escitalopram exposure with a structured search algorithm and eligibility criteria for inclusion. Exposure data from individual studies (N=16) were adjusted for between study differences not attributed to CYP2C19 genotype. The exposure changes associated with *CYP2C19*2, *3*, and **17* as compared with *CYP2C19*1* were pooled in meta-analyses fixed-effect and random-effects models. Publication bias and sensitivity analysis were also assessed by funnel plots and meta-regressions, respectively.

4.5.2 Results

Sixteen studies from 14 publications met the inclusion criteria. Eligible studies included 847 patients from psychiatric patient trials and 140 healthy subjects from pharmacokinetic studies. Compared to subjects with EM/EM (*CYP2C19*1/*1*) genotype, the exposure to (es)citalopram increased by 95% (95% CI 40-149%, $p<0.0001$) in the PM/PM (*CYP2C19*2/*2, *2/*3,* or **3/*3*), 30% (4-55%, $p<0.05$) in the EM/PM (*CYP2C19*1/*2* or **1/*3*), and 25% (1-49%, $p<0.05$) in the UM/PM (*CYP2C19*17/*2* or *17/**3*) groups. In contrast, the exposure to (es)citalopram decreased by 36% (27-46%, $p<0.0001$) in the UM/UM (*CYP2C19*17/*17*) and by 14% (1-27%, $p<0.05$) in the UM/EM (*CYP2C19*17/*1*) (Fig. 11).

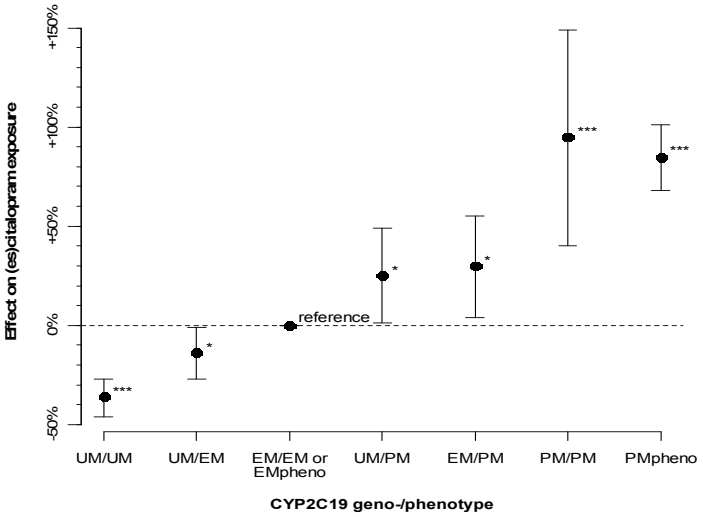

Figure 11 *The effect of CYP2C19 genotypes or phenotypes on (es)citalopram exposure. The filled circles represent the mean change (in %) in (es)citalopram exposure (using EM/EM as reference) and error bars 95% confidence intervals of the estimate. The dotted line indicates the exposure in the reference group (EM/EM). Asterisks indicate statistically significant differences between EM/EM and each of the 5 other genotype groups or between EMpheno and PMpheno groups, *p-value <0.05, ***p value <0.0001*

5 DISCUSSION

This thesis presents the findings related to CYP2C19 polymorphisms in 5 individual studies and also witnessed the progress that has been made over 15 years in the understanding of CYP2C19 polymorphism in relation to genetic variants, individual and population variability, genotype-phenotype correlation, and the impact on clinical practice.

In ***Study I***, we demonstrated that PMs of S-mephenytoin had significantly slower metabolism of omeprazole than IMs and EMs. The metabolic status was associated with the S-mephenytoin hydroxylation polymorphism. We identified heterozygous EM from family studies and homozygous EM based on a rapid mephenytoin S/R ratio (<0.05). Four years late Furuta *et al* [3] performed a similar study in Japanese but classified subjects by genotyping. The results from these two studies were comparable in PMs with respect to the mean AUC of omeprazole (14273 vs. 14778 nM•h), suggesting a good correlation between phenotyping and genotyping. Our result in EMs, however, was 41% lower than Furuta's, supporting the expectation for the rapid metabolisers with very fast mephenytoin S/R ratio of <0.05. More interestingly, the mean AUC of omeprazole in IM was also 35% lower in our study than Furuta's, suggesting a considerable difference between Caucasians and Japanese. This ethnic difference is consistent with other studies [95], reflecting lower CYP2C19 activity in Asians.

Study I is the first report, prior to any genotyping method available, demonstrating the interphenotype difference of CYP2C19 in pharmacologic effect of omeprazole by means of plasma gastrin levels. After multiple doses, the gastrin levels were elevated significantly in PM and IM but remained virtually unchanged in EM. The increase of gastrin AUC in PM and IM were in an omeprazole-concentration-dependent fashion. This finding was in line with Furuta *et al* [44] where they revealed cure rates of *Helicobacter pylori* infection and healing rates of gastric/duodenal ulcers in patients treated with omeprazole in a gene-dose-effect manner. In addition, ***Study I*** further analyzed the concentration ratio of omeprazole and hydroxyomeprazole at 3 hours post dose and demonstrated a significant difference with no overlap among the three phenotype groups. The results suggest that the ratio could be used as an index of CYP2C19 activity following a single dose of omeprazole as a probe.

The suitability of omeprazole as a probe for CYP2C19 was further investigated in ***Study II*** with a population of 160 unrelated Swedish subjects. The validation also included seven subjects who had previously been phenotyped as PM by the mephenytoin method in order to cover the low incidence of PMs in the population studied. The utility of omeprazole as a probe drug for CYP2C19 phenotype was validated through comparisons with S-mephenytoin hydroxylation phenotype and CYP2C19 genotype with respect to *CYP2C19*2*. The potential advantages of using omeprazole include 1) to address the concerns of using mephenytoin (unavailability and AEs); 2) to identify the subjects with very rapid hydroxylation based on a normal distribution of logMR of omeprazole; 3) might be useful as a dual substrate probe for both CYP2C19 and CYP3A4 activities.

Study II is the first report to systematically assess the correlation between MR of omeprazole and S/R mephenytoin ratio in a population study. Considering the assay utility and convenience in practice, we used a single blood sample collected at 3 hours post dose as a measurement, which was selected based on the results from *Study I*. Spearman rank coefficient of correlation appeared to be low (0.63, $p<0.001$) between MR of omeprazole and S/R mephenytoin ratio, likely due to 18% of subjects with undetectable S-mephenytoin value being assigned to S/R ratio of <0.05. In drug development process, omeprazole has already become a popular probe for CYP2C19 to assess the potential for drug-drug interaction. In most of the cases, series of blood samples are collected and AUCs of omeprazole are compared when dosed alone vs. in combination with the drug in question. Omeprazole can be used as a single probe and in cocktail studies as exemplified in Karolinska cocktail [37] and Cooperstown cocktail [147]. It is also successfully used as a dual substrate probe to evaluate induction of CYP2C19 and CYP3A4 in an efavirenzin study [148].

We also used omeprazole as a dual substrate probe to estimate the induction potential of carbamazepine (CBZ) in ***Study III*****.** CBZ is a known potent inducer of CYP3A4 that leads to the increase of clearance of CBZ and other drugs, for example oral conceptives [149]. Intuitively in our study, the mean AUC of omeprazole sulphone increased by 44% after omeprazole was administered concomitantly with CBZ, confirming that CBZ induced the formation of omeprazole sulphone mediated by CYP3A4. The ratio between the AUCs of omeprazole and sulphone decreased in all 5 subjects, but failed to show statistical significance ($p=0.052$). A large variation was found among the 5 patients, two (No 3 and

5) had dramatic reduction while others showed marginal effects. The small sample size, in addition to the variation, contributed largely to the low statistical power.

The mechanism of enzyme induction caused by CBZ is less studied. One report from Luo and coworkers demonstrated CBZ weakly activated PXR and induced CYP3A4 activity as compared to rifampin in the human hepatocytes system [150]. Enzyme induction occurs when a ligand (CBZ in this case) binds to PXR or CAR, trigging RNA polymerase and mRNA transcription. The process could be less enzyme-specific and nonselective. Thereby the potential of CBZ for inducing CYP2C19 (hydroxylation of omeprazole) cannot be completely ruled out. In ***Study III***, both AUCs of omeprazole and hydroxyomeprazole decreased by ~ 40% after coadministration with CBZ, resulting in an unchanged ratio between the parent and metabolite. The unchanged ratio suggested that CBZ had less or no effect on the formation of hydroxyomeprazole. This finding is actually in line with a number of studies reporting that the CBZ-mediated induction was found only with CYP3A4, CYP1A2, and P-glycoprotein [151], [150]. Since CYP3A4 and CYP2C19 are both involved in the primary and secondary metabolism of omeprazole as shown in Figure 3, the precise contribution of CYP3A4 induced by CBZ is, however, difficult to establish in our study.

The oral anticoagulant warfarin is widely used for the treatment and prevention of thromboembolic disorders. Because it has a narrow therapeutic index, more than 10-fold interindividual variability in dose requirement, and multiple drug interactions, the clinical use of warfarin is challenging. The goal of pharmacogenetic testing is to aid clinicians in prescribing the right drug, with the right dose, at the right time. It is crucial that major functional allelic variants of CYP450 genes have been identified and included in the testing. In our earlier study where influence of *CYP2C9* and *CYP2C19* genetic polymorphisms on warfarin maintenance dose and metabolic clearance was evaluated, no significant effect of *CYP2C19* genotypes on the clearance of unbound R-warfarin was found [141]. At the time of that study, the *CYP2C19*17* allele had not yet been described, **17* alleles thus being classified as *CYP2C19*1*. Adding *CYP2C19*17* genotyping to ***Study IV***, we found a significant effect of *CYP2C19* genotype on R-warfarin clearance, carriers of *CYP2C19*17* having, on average, 32% higher clearance than carriers of *CYP2C19*2*. ***Study IV*** is to our knowledge the first study to include the gain-of-function allele in the analysis of the effect of *CYP2C19* genotypes on the enantioselective pharmacokinetics and INR response of warfarin.

Patients with two functional *CYP2C19*1* alleles had clearance values in between the *CYP2C19*17* and *CYP2C19*2* groups. Analysis of the**17* allele now allowed identification of a subgroup of patients with a predicted, on average, higher CYP2C19 activity as compared to those carrying two *CYP2C19*1* alleles. The improved prediction of the phenotype together with a larger patient population is a probable explanation to the observed significant effect of the genotype on R-warfarin clearance. Similar to this study, a significant effect of *CYP2C19*17* on the pharmacokinetics of other CYP2C19 substrates has been shown, exemplified by omeprazole [152] and escitalopram [153] .

It is interesting that using single nucleotide polymorphisms (SNPs) of loss-of-function alleles to predicate phenotypes for CYP2C19 seems to be well established compared to that of gain-of-function alleles (*CYP2C19*17)* where the controversy exists. A quantitative review by Li-Wan-Po A and colleagues displayed a large overlap in PK variables between carriers of **1/*1* and **1/*17* (similar results were also observed in our studies), and a modest effect between carriers of two gain-of function alleles (*CYP2C19*17/*17*) and two loss-of-function alleles (PMs). They questioned the utility of *CYP2C19*17* in practice, suggesting to assign *CYP2C19*17* homozygotes as EM rather than ultrarapid metabolisers (UM) [154].

We also explored the *CYP2C* gene cluster haplotypes comprising the clinically most important *CYP2C* variants in ***Study IV***. The frequencies higher than 10% in our Italian population were the same as those previously reported in Nordic populations [155]. Consistently, *CYP2C19*17* was observed with an allele frequency of 17% and in strong linkage disequilibrium (LD) with *CYP2C9*1* and *CYP2C8*1*. This result supports the observed correlation between *CYP2C19*17* and R-warfarin clearance being independent from the other SNPs assessed in this study. Our analysis also confirmed the moderately strong LD between *CYP2C8*3* and *CYP2C9*2* (D'=0.78).

In addition to the effect of *CYP2C19* genotypes on R-warfarin clearance, an association with warfarin response was observed in ***Study IV***, using INR/daily dose as a marker. *CYP2C19* genotypes accounted for 7% of the variance in INR/daily dose. Genetic (*VKORC1*, *CYP2C9*, and *CYP2C19*) and non-genetic (age, gender, and body weight) covariates together explained 52% of the variability. Although the impact of *CYP2C19* genotypes was much smaller than that of *VKORC1* and *CYP2C9*, it was nevertheless a significant factor. This finding is in line with the results of a recent PK/PD study [156],

suggesting that the R-enantiomer does indeed contribute to the anticoagulant effect of warfarin, based on both separate and combined administration of pure warfarin enantiomers.

Meta-analysis is a powerful approach to give a thorough summary of several studies that have been conducted on the same topic. ***Study V*** is the first meta-analysis based on a systematic review of accumulated information that addresses the relationship between *CYP2C19* genotypes and the exposure to citalopram or escitalopram. Compared to *CYP2C19*1/*1,* the exposure to (es)citalopram decreased significantly ($p<0.0001$), by 36% (95% CI, 27-46%) in *CYP2C19*17/*17*. The precise estimate was derived from pooled data of 4 studies with 36 subjects homozygous for *CYP2C19*17* and 237 homozygous for *CYP2C19*1* and assessed to be reliable based on the funnel plot asymmetry inspection. However, it is to be noted that there was a considerable heterogeneity among studies, indicating that the different results in individual studies may partly reflect differences in study populations and study designs. Taken together, our data from the meta-analysis demonstrates that homozygous carries of *CYP2C19*17,* on average, achieved 36% lower exposure to (es)citalopram, and may need higher doses to reach an exposure similar to that in subjects homozygous for *CYP2C19**1 .

Explicitly discussing the limitation would help interpret study findings appropriately. In our study, we could not account for the use of potential interacting drugs or the role of other CYP enzymes (such as CYP2D6) involved in citalopram or escitalopram metabolism. Co-medication was an exclusion criterion in the studies performed in healthy subjects, but was sometimes allowed in the patient studies. The drugs used concomitantly in patient studies were rarely specified clearly. Since drug interactions may influence the PK of citalopram or escitalopram, the possible impact of drug interactions cannot be excluded as a source of interindividual variability.

Data extraction, categorization, and evaluation for eligibility are critical elements for an unbiased, transparent, and valid result. Recently, 3 meta-analyses with the same focus of CYP2C19 polymorphism on clinical outcome of clopidogrel treatment (published by Bauer *et al* [122], Holmes *et al* [123], and Jang *et al* [157], respectively) presented interesting outcomes. Three groups used almost identical searching strategy and study inclusion/exclusion criteria, so that the numbers of studies/patients included in the final analyses were similar, i.e. 15/19328, 16/20785, and 32/42016 in Bauer's, Jang's, and

Holmes's study, respectively. The first two analyses have >75% of studies overlapping (12/15 vs. 12/16), but reached opposite conclusions. Holmes' meta-analysis covered 100% and 94% of Bauer's and Jang's studies, respectively, demonstrated an overall negative result of cardiovascular events, supporting the conclusion of Bauer *et al*. When performing a systemic review, it is extremely challenging, and sometimes tricky, to assign grades to non-continuous variables in efficacy or safety outcomes during data extraction process.

Interpretation of findings to provide appropriate implications for practice is another challenge. Based on our pooled analysis, we believe the results aid in understanding the interindividual variability in the exposure to citalopram and escitalopram in psychiatric patients and facilitate dose selection particularly for the homozygous carriers of loss-of-functions (*CYP2C19*2* or **3*) and the gain-of-function (*CYP2C19*17*) alleles. The findings could improve individualization of citalopram or escitalopram therapy and could also be used for physiologically-based pharmacokinetic (PBPK) modeling as well as PK/PD modeling. However due to the difficulty in accurately measuring PD response in depression and anxiety disorders, a clear relationship of concentration-response cannot be simply established for citalopram and escitalopram as it can for dose-concentration relationships. In addition, the contribution of a specific enzyme may vary substantially between drugs and the quantitative influence of individual polymorphisms may theoretically be substrate specific. Therefore, it would be premature to extend the findings of (es)citalopram exposure in the specific allelic combination genotypes to a more general model of CYP2C19 activity prediction.

6 FUTURE PERSPECTIVES

The clinical utility of genotyping and personalized medicine is generally believed to be favored when the drug has a narrow therapeutic window or is catalyzed predominantly by a polymorphic enzyme. Theoretically, genotyping the enzyme helps to predict therapeutic failures or AEs, and potentially speeds up the selection of optimal dosage range for individual patients. However, the promises of new technology are not translated into appreciable improvement in patient care as yet. The clinical uptake of pharmacogenetic or pharmacogenomic testing including genotype-guided prescribing is slow.

To overcome the myriad obstacles, it requires joined-efforts from basic research, translational medicine, clinical laboratory medicine, clinical pharmacology, and regulatory oversight to move the new technology from bench to bedside.

Basic research- A large body of information on the characterization of drug-mobilizing enzyme/polymorphisms has been generated. The gaps exist, however, in our knowledge regarding how to utilize the information to explain interindividual variability and to predict drug response outcomes. CYP genotype alone in many cases cannot be the answer. The complexity of biological systems and disease status should be considered. It is unclear if common diseases (e.g. diabetes, asthma, heart diseases, and cancer) could change the drug metabolism pattern through making up the genes of drug-metabolizing enzymes. Concerning pharmacogenetics and pharmacogenomics, it is necessary to study polymorphism in trans-acting genes or in the regulatory genes involved in transcriptional regulation, or receptor polymorphism. The “private” mutation, rare mutations in various populations and unknown rare polymorphism will add knowledge substantially to the current understanding.

Clinical-laboratory-medicine/Translational medicine- Clinical laboratory research is an important piece between basic sciences and clinical practice. The transition from bench to bedside may not happen anywhere without the involvement of clinical labs with adequate knowledge, even though there are fruitful basic research and regulation. Concerning the predictive genotype for P450s in the clinic, it does not occur routinely for many reasons including medical, legal, economic, social, ethical, and organizational

issues [158]. Of these, lacking solid evidence for their advantage over current medical practice is the key barrier.

To bridge the gap, randomized, large, conclusive, prospective studies showing improvement of drug efficacy following genotyping would be ideal but impractical. We cannot expect that such studies will in fact be performed for every new biomarker that will be discovered. A more pragmatic approach should be considered. In current clinical research, genotyping variants from the core and extended ADME gene list are recommended as exploratory objectives. For DDI and ADME studies, the genotyping tests are included as part of standard requirement and results are typically used within the study specific context. If there is significant impact of PM on the candidate drug, the information will be captured in its labeling. However, integrated use of the genotype data comprehensively and accumulatively across studies has not yet been a standard analysis. Since DDI and ADME are typically performed in healthy subjects, the disease related information is missing from the current practice.

Validation of new biomarker/genotype requires tremendous effort, may need to include the *in vitro* novel methods, model-based simulation/prediction, tissue banks, and clinical trial designed with well defined inclusion and exclusion criteria including possible interactions with the drug studied. Generating data from clinical trials to demonstrate the associations between biomarkers and efficacy outcomes can be time consuming and costly. The innovative study designs, including adaptive clinical trials, provide considerable advantages. Typically, an adaptive study design would allow interim analyses in order to 1) stop/adjust patient accrual, cohorts or dose(s); 2) revise the hypotheses; and 3) stop the trial early for success, futility or harm. It is anticipated that use of adaptive study design could accelerate the turnaround time of a large clinical trial with multiple objectives/hypotheses.

Other barriers in pharmacogenetic application include lack of education for health professions and the need to develop evidence-based clinical practice guidelines on the testing.

Regulatory oversight- Regulatory structure needs to support the growth of pharmacogenetic/pharmacogenomic testing. According to the FDA, about 10% of labels for FDA-approved new drugs contain pharmacogenomic information [159]. It sounds like a small portion, but represents a substantial increase since the 1990s. Regulatory

agencies need to ensure the quality of products, especially when the results are used in making major medical decisions. As a matter of fact, some commercial laboratories (mainly in US) are broadly marketing their lab-based complicated genetic testing which they do not have the knowledge to fully interpret. The balance point between protecting patients and encouraging innovation can be a challenge for regulatory agencies.

In Europe, there are no harmonized regulations or central regulators for medical diagnostics/biomarkers. The regulation can only occur at the member-state level. It is possible that clinical utility of approved tests may turn out to be no longer maintained at the current rate or level, whereas non-approved tests are used in practice [160]. In order to protect patients and give clinicians' confidence on personalized medicine and pharmacogenetic/pharmacogenomic testing; it is more important than ever to call for regulatory support and revolution.

To summarize, genotype-guided prescriptions, including algorithms, are being applied to a few cases and will hopefully increase in coming years. In order to bridge the gap between research and clinical practice, it will be crucial to accelerate testing the clinical validity of pharmacogenetic markers, to train medical professionals, and to deliver the clinical benefit of new biomarkers to patients.

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